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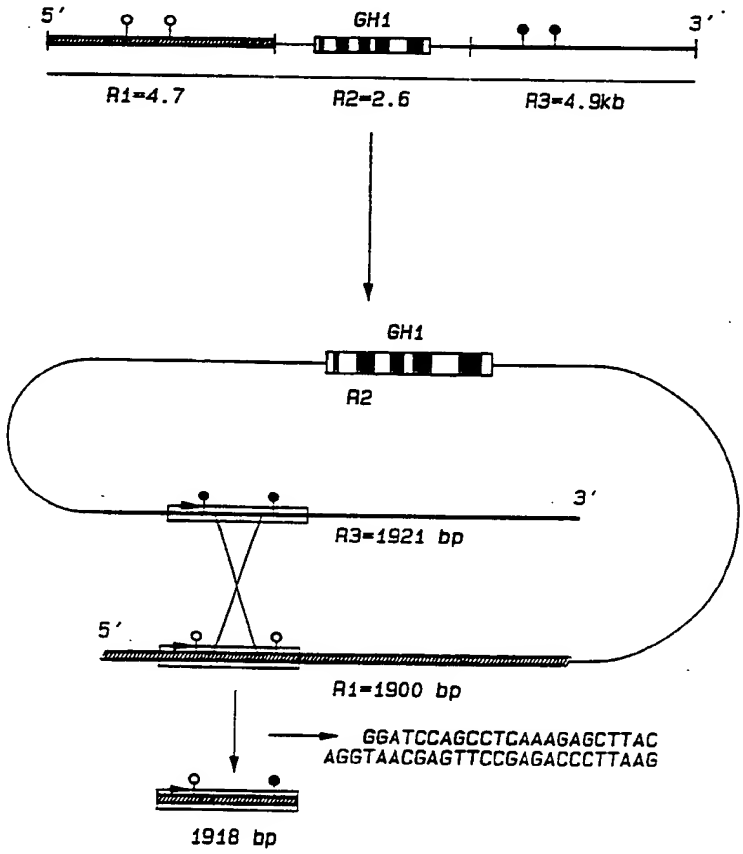
(54) Title: MOLECULAR DETECTION OF GENE DELETIONS

(57) Abstract

A method of detecting gene deletions includes the steps of obtaining a genomic DNA sample possibly including a gene deletion, the gene having two ends normally flanked by two homologous regions having substantially identical portions, the gene deletion being in the form of a fusion fragment of the two homologous regions. The genomic DNA is amplified using a set of primers capable of amplifying both the fusion fragment and the two normal homologous regions having the gene in-between. The amplified fusion fragments are characterized alone indicating a homozygous deletion, a combination of fusion fragments and the two homologous regions and the gene in-between indicating a heterozygote deletion, or the two homologous regions and the gene alone indicating a homozygous nondeletion. Novel primers for use in the inventive method are also disclosed.



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MOLECULAR DETECTION OF GENE DELETIONS

This application is a continuation-in-part of Serial No. 530,218, filed May 30, 1990.

TECHNICAL FIELD

This invention relates to a method of detecting gene deletions and primers used with the method. More specifically, the present invention can be used for the detection of growth hormone gene deletions, such information being used to identify families at risk for recurrence of growth hormone deficiency as well as providing more accurate estimates of the risk for affected individuals to develop immune intolerance to treatment with exogenous growth hormone.

BACKGROUND ART

Molecular biologists have utilized processes for amplifying existing nucleic acid sequences if they are present in a test sample and detecting them if present by using a probe. The U.S. patents 4,683,195 to Mullis et al., issued July 28, 1987, 4,800,159 to Mullis et al., issued January 24, 1989, 4,683,194 to Saiki et

al., issued July 28, 1987 and 4,889,818 to Gelfand et al., issued December 26, 1989 all relate to processes for amplifying, detecting, and/or cloning nucleic acid sequences and a purified thermostable enzyme used therewith. The Saiki et al patent further relates to a method for detecting polymorphic restriction sites and nucleic acid sequences. Each method utilizes probes, nucleic acid being hybridized to the probe. The Saiki et al patent utilizes subsequent digestion with restriction enzymes that cleave those oligomers that have hybridized the nucleic acid and reform the restriction site. The resulting cut and uncut labeled oligomers are separated and detected based on the type of probe label.

The previously mentioned patents relate to amplification of nucleic acid sequences but do not at all relate to situations where the nucleic acid may be in the form of fusion fragments. The present invention provides a process for detecting gene deletions in the form of fusion fragments and primers which can be used in the process. In other words, the present invention relates to the detection of gene deletions in specific forms of genomic DNA not addressed by

the prior art. For example, the deletion of a structural enzyme for the systemic manufacture of growth hormone results in a fusion fragment which can be detected pursuant to the present invention.

Human growth hormone deficiency has a prevalence of between 1 in 4,000 and 1 in 15,000 and 5% to 30% of the cases are estimated to be familial (1,2). The molecular basis of one Mendelian form, isolated growth hormone deficiency type 1A (IGHD1A), results from the deletion of the structural gene for growth hormone (GH1) (3). Treatment of the associated, severe dwarfism that presents itself in early infancy is frequently complicated by the development of anti-growth hormone antibodies following treatment with exogenous growth hormone (3,4). Affected individuals are thus at an increased risk to develop immune intolerance to exogenous growth hormone and their siblings are at a 25% risk for also being affected by the disorder.

With regard to nucleic acid amplification, the polymerase chain reaction amplification technique provides an alternative to Southern blotting for the identification of a

growing number of genetic disorders. To facilitate and simplify detection with IGHD1A, applicant has developed a rapid method utilizing a modified polymerase chain reaction amplification technique followed by restriction enzyme digestion and visualization of the DNA fragments that enables identification of carriers (hetrozygotes and homozygotes) for the GH1 gene deletions as well as noncarriers. It is important to detect homozygotes for the GH1 gene deletions because such patients having never systemically produced growth hormone are immune reactive to growth hormone replacement therapy. These patients produce antibody to the growth hormone given clinically and do not react to such therapy. It is important to detect heterozygotes because the mating of two heterozygotes have a 25% chance of producing a homozygous offspring.

The GH1 gene deletion is in the form of a particular structural type of genomic DNA. The GH1 gene and its immediate flanking sequences from normal individuals are contained within three EcoRI derived fragments of 4.7, 2.6, and 4.9 kb which are referred to as R1-3, respectively (7). Within R1 and R3 are homologous regions of DNA that contain either a

BglI and HaeII site (R1) or two SmaI sites (R3). Unequal recombination between these two homologous regions yield fusion fragments that contain a single SmaI site and cause 6.7 kb deletions of genomic DNA that contain GH1(8). Amplification of such fusion fragments and the use of such amplification to detect a gene deletion are not addressed by the prior art. Further, digestion of these fusion fragments utilizing specific enzymes to which the sites are susceptible to determine heterozygotes, homozygotes, and noncarriers is also not addressed by the prior art.

The present invention provides a method for detecting gene deletions in the form of the above discussed fusion fragments, heterozygotes or carriers of the deletions, and noncarriers.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method of detecting gene deletions, the method including the steps of obtaining a genomic DNA sample possibly including a gene deletion, the gene having two ends normally flanked by two

homologous regions having substantially identical portions, the gene deletion being in the form of a fusion fragment of the two homologous regions. The genomic DNA is amplified using a set of primers capable of amplifying both the fusion fragment and the two homologous regions having the gene in-between. The amplified fusion fragments are characterized alone indicating a homozygous deletion, a combination of fusion fragments and the two homologous regions having the gene in-between indicating a heterozygous deletion, or the two homologous regions and the gene alone indicating a homozygous nondeletion.

The present invention further provides a primer set for detecting gene deletions in a genomic DNA sample possibly including a gene deletion, the gene having two ends normally flanked by two homologous regions having substantially identical portions, the gene deletion being in the form of a fusion fragment of the two homologous regions. The primer set includes means for amplifying both the fusion fragment and the two homologous regions having the gene in-between.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 is a photomicrograph of ethidium bromide staining patterns of DNA fragments obtained by PCR amplification following subjection to digestion with SmaI and polyacrylamide gel electrophoreses: lane 1-control, lanes 2 and 3 known GH1 gene deletion heterozygote and homozygote, respectively, lanes 4 and 5 parents of subjects 1-2 (Table 1), and lanes 6 through 12 subjects 1-7 (Table 1), squares representing males and circles females;

Figure 2 is a schematic representation of the three EcoRI derived fragments that flank (R1 and R3) or contain (R2) the GH1 gene (top), the location of the 1900 and 1921 bp genomic fragments that are obtained by PCR amplification using the indicated oligonucleotide primers being shown in the middle (noting the first three 5'

bases of both oligonucleotides being added to facilitate cloning) the 1918 bp fusion fragments obtained from genomic DNA of heterozygotes or homozygotes for GH1 gene deletions shown below, circles indicating positions of recognitions sites for BalI (open) HaeII (stippled or SmaI (solid);

Figure 3 shows three different sizes of deletions which encompass the human growth hormone gene of humans detected by Southern blot analysis of DNA from individuals with familial isolated growth hormone deficiency type 1A as well as the characteristic fusion fragments that are associated with each. The numbers on the right indicate th size of fragments obtained after digestion with various restriction enzymes, as set forth above regarding Figure 2;

Figure 3 is a schematic representation of the 3 EcoRI-derived fragments that flank (R1 and R3) or contain (R2) the GH-1 gene. The sizs and relative loctions of 6.7, 7.0 and 7.6 kb deletions are shown by solid, open and stippled bars. Using oligonucleotide primers (indicated by small horizontal arrows) the sizes and restriction patterns of resulting PCR

amplification products obtained from control and deletion DNAs are shown on the right;

Figure 4 is a photomicrograph of ethidium bromide-staining patters of DNA fragments obtained by PCR amplification of genomic DNAs and polyacrylamide gel electrophoresis. Lanes 1, 2 and 4 GH-1 deletion homozygotes, lanes 3 and 5 GH-1 deletion heterozygotes, lanes 6 and 7 controls lane 8 blank and lane 9 molecular weight marker;

Figure 5 is a photomicrograph of ethidium bromide-staining patters of DNA fragments obtained by PCR amplification of genomic DNA using the ANF primers and polyacrylamie gel electrophoresis. Lanes 1 and 22 contain molecular weight markers. Patterns obtained from 5 controls are shown in lanes 2-6, 7-11, 12-16 and 17-21 following digestion with MspI, DdeI, HaeIII or RsaI, respectively; and

Figure 6 shows aliquots of PCR products following amplification of the human prion gene were subjected to polyacrylamide gel electrophoresis and visualized after staining wit ethidium bromide. Lanes 1 through 6 represent amplified products from members of the probands's family at risk for developing Creutzfeldt-Jakob

disease. Lane 7 is the proband and lane 8 is a normal control.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there is provided a method of detecting gene deletions. Generally, the method includes the steps of obtaining a genomic DNA sample possibly including a gene deletion, the gene having two ends normally flanked by two homologous regions having substantially identical portions, the gene deletion being in the form of a fusion fragment of the two homologous regions. The genomic DNA is amplified using a set of primers capable of amplifying both the fusion fragment and the two homologous regions having the gene in-between. Amplified fusion fragments are characterized alone indicating a homozygous deletion, a combination of fusion fragments and the two homologous regions and the gene in-between indicating a heterozygote deletion or the two homologous regions and the gene alone indicating a homozygous nondeletion or noncarrier. Primers are provided which are capable of amplifying either the fusion fragments

or the two homologous regions having the gene in-between thereby being capable of detecting either the homozygote, the carrier heterozygote, or the noncarrier homozygote nondeletion.

Unlike prior art methods, this method provides detection of gene deletions wherein the gene deletion is in the form of fusion fragments. The fusion fragments are derived from the gene that is otherwise deleted having the two homologous regions on each side thereof. The two homologous regions have substantially identical portions which can fuse as a result of the gene deletion.

The genomic DNA sample would normally be obtained from a patient desirous of a genetic analysis assessing the risk of carrying a gene deletion of the type which produces fusion fragments of the flanking genetic material. An example of such a deletion is the deletion of the GH1 gene of the R2 region of EcoRI.

As shown in Figure 2, the GH1 gene is contained in the R2 region and is immediately flanked by R1 and R3. R1 and R3 are homologous regions of DNA that are believed to be of evalutionarily related.(7) The two homologous regions have areas of substantially similar

nucleotide sequence. Where a deletion occurs of the R2 region, there is unequal recombination between these two homologous regions yielding the fusion fragments that contain a single SmaI site and cause the 6.7 kb deletions of genomic DNA that contain the GH1 region, as shown in Figures 2 and 3.

The amplification of the genomic DNA is performed using the polymerase chain amplification method as described by Saiki et al with modifications (6). However, a particular set of primers are used which are capable of amplifying both the fusion fragment and the two homologous regions having the gene in-between. The set of primers are complementary to the substantially identical portions of the two homologous regions. This enables the primers to amplify either the fusion fragments or the two homologous regions having the gene in-between. That is, the set of primers are capable of cloning regions R1 and R3 in the fusion fragment form or having the R2 region therebetween. It is the capability of these synthetic oligonucleotide primers to specifically bind and amplify the fusion fragments or the undeleted R1-3 sequence that provides the present invention with the

capability of detecting either a homozygous deletion, a heterozygote having deletion and nondeletion DNA strands or the homozygote having no deletions in either DNA strand.

Specifically, the oligonucleotide primers used for PCR amplification in accordance with the present invention are shown in Figure 2. Each primer sequence of the primer set includes a nucleotide triplet at each 5' end thereof to facilitate cloning of the polymerase chain reacting amplification products. That is, the ends of the amplified products contain sequences that enable them being inserted into plasmid or phasge vectors to facilitate propagation, DNA sequencing or expression vector analysis. Specifically, primer sequence (a) shown in Figure 2 includes a triplet 5'GGA3' flanking the 5' end thereof. Primer sequence (b) shown in Figure 2 includes the triplet 3'AAG5' flanking the 5' end thereof.

Other synthetic oligonucleotide primers can be constructed for the detection of other deletions wherein the deletion results in a fusion fragment of homologous flanking nucleotide segments. The commonality of these primers is their complementary structure to the

substantially identical portions of the two flanking homologous regions.

In accordance with a more specific characterizing aspect of the inventive method, at least one the homologous regions of the DNA sample contains a site susceptible to cleavage by a particular enzyme. In this situation, the characterizing step of the present invention would be further defined as enzymatically digesting the amplified genomic DNA with a particular enzyme, and producing digestion fragments from the fusion fragments having lengths different from the digestion fragments of the two homologous regions having the gene therebetween. The samples would then be identified as including deletions based on observing the different size digestion fragments. Identification can be performed by electophoretically separating the produced fragments and visually observing the separated fragments.

More specifically, with regard to the GH1 gene deletion, and as set forth previously, the GH1 gene is from the R2 region cleaved by EcoR1. Digestion with EcoR1 also produces the two homologous flanking regions R1 and R3. The

R3 region includes two SmaI sites. It is known that the unequal recombination between the two homologous regions yield fusion fragments that contain a single SmaI site and cause the 6.7 kb deletions of genomic DNA that contain GH1 (8), as shown in Figures 2 and 3. Accordingly, digestion with the restriction enzyme SmaI of the fusion fragments having the single SmaI site produces fragments of different lengths than digestion of the two homologous regions having the otherwise deleted gene in-between, this segment having two SmaI sites and the 6.7 kb genomic DNA that contains GH1.

After digestion using the restriction enzyme SmaI, a 1918 bp fusion fragment is amplified from a homozygote for GH1 deletion, 1900 and 1921 bp fragments are amplified from a nondeletion control, and a combination of the 1918 bp and 1900 bp and 1921 bp fragment is amplified from a heterozygote. Digestion produces 1900, 761, 712, and 448 bp digestion fragments from the control, a 1470 and 448 bp digestion fragments from the homozygote, and the combination of the 1900, 761, 712, and 448 bp with the 1470 and 448 bp digestion fragments from the heterozygote. Electrophoreses of the

different samples results in different patterns based on the combinations of the different length fragments. Thusly, the nondeletion homozygote control, the homozygote deletion carrier and the heterozygote carrier of the deletion on a single DNA strand can be identified.

EXPERIMENTAL EVIDENCE

Experiment 1

Seven subjects with severe growth retardation due to isolated GH deficiency (IGHD) were studied. Diagnostic criteria included severe growth retardation with height greater than -5 SD, decreased growth rate, retarded bone age, normal karyotype and T4 and peak GH levels less than 7 ng/ml after various provocative stimuli (see Table 1).

DNA Isolation

High molecular weight nuclear DNA was isolated from peripheral leukocytes of subjects, selected relatives and controls as previously described (5). The concentration of each sample was determined by measuring the optical density of the purified DNA at 260 nm.

DNA Amplification and Restriction Endonuclease Analysis

DNA amplifications were performed using the polymerase chain reaction (PCR) amplification method as described by Saiki et al with modifications (6). Reaction mixtures had a total volume of 100 ul and contained 100 to 200 ng of high molecular weight genomic DNA, 1 uM of each synthetic oligonucleotide primer (see Figure 2), 200 uM each of dATP, dGTP, dCTP and TTP, 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM MgCl2, 0.01% gelatin and 2.5 units of Taq polymerase. The samples were overlaid with mineral oil to prevent condensation and placed in a Perkin Elmer Cetus thermocycler. Template DNA was denatured at 94'C for 6 minutes and subjected to 30 amplification cycles. Each cycle consisted of a 30 second DNA denaturation period at 94'C, 30 second DNA annealing period at 60'C and a 2 minute DNA extension period at 72'C. Following the 30 cycle amplification process, a 10 minute extension period at 72'C was performed.

Following amplification, 25 ul of each reaction mixture was digested with the restriction endonuclease SmaI according to the specification of the supplier. The buffer

contained 20mM KCl, 6mM Tris-HCl (pH 8.0), 6mM MgCl2, 6mM beta-mercaptoethanol, 100 ug/ml bovine serum albumin and 12 units of enzyme. The reaction was allowed to proceed at room temperature for 2-1/2 hours. The SmaI digested PCR products were visualized following electrophoresis on a 5% polyacrylamide gel.

Results

The patterns obtained following ethidium bromide staining of the PCR products subjected to digestion with SmaI and electrophoresis in a polyacrylamide gel are shown in Figure 1. The patterns obtained using genomic DNA from subjects 3-7 (Table 1) seen in lanes 8-12 were identical to that of a normal control (lane 1) and had fragments of 1900, 761, 712 and 448 bp in length. Patterns of subjects 1-2 (lanes 6 & 7) and a known IGHD1A subject (lane 3) differed in only having fragments of 1470 and 448 bp. DNA from the father (lane 4) and mother (lane 5) of subjects 1-2 and a known carrier of IGHD1A (lane 2) differed from the control in having additional fragments of 1470 bp which were also seen in samples from subjects who were

homozygous for GH1 gene deletions (lanes 3,6 and 7).

As shown in Figure 2, PCR amplification of the fusion fragments using the oligonucleotide primers of the present invention yielded 1918 bp fragments. These differed from the PCR derived fragments obtained from the DNA controls which were 1900 and 1921 bp in length, corresponding to R1 and R3 sequences, respectively. Following digestion with SmaI, the 1900 bp fragments (R1 sequences) were not cleaved, as these fragments do not include restriction sites susceptible to the enzyme as shown in Figure 2. The 1921 bp fragments including the R3 sequences were cleaved to yield three fragments of 761, 712 and 448 bp corresponding to the three segments separated by the two SmaI sites.

In contrast, following digestion with SmaI, the 1918 bp PCR fragments derived from homozygotes for the GH1 deletion yielded fragments of 1470 and 448 bp as shown in Figure 1, lanes 3, 6, 7. These two fragments results from the presence of the single SmaI site in their 1918 bp fusion fragment as shown in Figure 2. The PCR derived DNA fragments from a known

carrier (heterozygote) of the GH1 gene deletion yield a distinctive pattern following digestion with Sma1, as shown in lane 2 in Figure 1. In this case, the normal fragments (1900, 761, 712, and 448 bp) derived from the R1 and R3 sequences on their normal chromosome are seen as the 1470 and 448 bp fragments derived from the fusion of R1 and R3 that is associated with GH1 deletion.

Thusly, genotypes of subjects 3-7 are GH1/GH1 homozygotes while those of subjects 1 and 2 are deletion/deletion homozygotes while that of both their parents is GH1/deletion (heterozygous carriers), consistent with the above analysis (Table 1).

The above results were confirmed by Southern blot analysis in which the 3.7 kb BamHI derived fragments containing GH1 were normal (subjects 3-7); absent (subjects 1 and 2); or decreased in intensity as compared to normals (mother of subjects (Table 1) (3).

There are 38 reported cases of IGHD1A (3, 4, 9-13). While all have had severe growth retardation, many of the early cases reported were studied because they developed anti-growth hormone antibodies following treatment with exogenous growth hormone (3, 4, 9).

Subsequently, a growing number of cases have been detected in which immune intolerance following growth hormone replacement has not developed (8, 10-13). These later findings suggest that a significant proportion of severe cases of IGHD, regardless of their response to therapy, could be due to GH1 gene deletions.

In the cohort of 7 subjects studied with heights delayed greater than -5 SD, 2/7 (29%) were homozygous for GH1 gene deletions. Among subjects with onset of growth retardation before six months of age (subjects 1, 2 and 6) 2/3 (67%) had GH1 gene deletions (see Table 1). Applicants' finding that a significant proportion of such cases have IGHDIA agrees with the recent report of Parks et al who found that 5/13 (38%) with height deficits greater than -4 SD and 5/9 (56%) who also had peak growth hormone levels of less than 4 ng/ml following growth hormone releasing hormone administration had GH1 gene deletions (13). If the results of Parks et al are pooled with applicants then 7/20 (35%) of the severely growth retarded (greater than -4 SD) cases studied with IGHD have GH1 gene deletions.

The present method for detecting GH1 deletions provides an easier and more rapid

alternative to Southern blot analysis. Application of this method could provide prenatal diagnosis of at risk pregnancies such as future children of the parents of subjects 1 and 2 (see Table 1 and Figure 1) using DNA from amniotic fluid or chorionic villus cells. Furthermore, this method could facilitate studies to determine the true incidence of IGHD1A among children with severe growth hormone deficiency of early onset. Detection of such cases would, in turn, identify families at risk for recurrence as well as provide more accurate estimates of the risk for affected individuals to develop immune intolerance to exogenous growth hormone.

Experiment 2

Materials and Methods

Genomic DNA was isolated as previously described from individuals with GH1 gene deletions of 6.7, 7.0 and 7.6 kb in size.

DNAs were PCR amplified in 100 μl reactions containing mixtures of 400 μg of genomic DNA, 1 μM of each oligonucleotide primer used in experiment 1,200 μM each of dATP, dGTP, dCTP and TTP, 50 m KC1, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 0.001% gelatin and 2.5 units of TaqI

polymerase. The reactions were placed in a thermocycler (Perkin Elmer Cetus, Norwalk, Conn.), denatured at 90°C for six minutes, then subjected to 30 amplification cycles. Each cycle consisted of a thirty second DNA extension period at 72°C. After the 30 cycles the samples were allowed to anneal for 10 minutes at 72°C, then cooled to 0°C.

Following PCR amplification 25 μl of each reaction mixture were digested with the restriction endonucleases SmaI, BglI or HaeII using conditions specified by the commercial supplier (New England Biolabs). The resulting DNA fragments were then subjected to electrophoresis in 5% polyacrylamide gels and visualized by ethidium bromide staining (see Figure 3).

Results:

At least 3 different sizes (6.7, 7.0 and 7.6 kb) of deletions which encompass the human growth hormone (GH) gene of humans have been detected by Southern blot analysis of DNA from individuals with familial isolated GH deficiency type 1A. It is likely that these deletions resulted from unequal crossover events between the homologous geions which flank the GH

gene. Phenotypic heterogeneity in response to exogenous GH treatment is suggested in that 6/7 (86%) of subjects with 7.6 kb deletions responded well while 82% of all IGHD IA subjects studied have responded poorly due to development of anti-GH antibodies.

To determine if molecular heterogeneity could be detected in DNA from subjects with different size deletions, the DNA sequences that flank the GH were analyzed following polymerase chain reaction (PCR) amplification. Digestion patterns of PCR amplification products of different IGHD type IA subjects with the restriction endonucleases SmaI, BGlI, and HaeII showed characteristic differences for 9 subjects with deletions (4-6.7, 2-7.0 and 3-7.6 kb) (see Figure 3). In all cases, the location and size of the GH gene deletions found by analysis of PCR products agreed with previous estimates determined by Southern blot analysis. Interestingly, differences in development of high titers of anti-GH antibodies and poor growth response following GH treatment are unexplained, since different outcomes were observed in patients who had deletions of the same size and approximate location.

Experiment 3

Materials and Methods

Recently applicants have simplified detection of fusion fragments associated with GH1 gene deletions. To do this primers in R1 and R3 were selected that correspond to nucleotides 1337-1363 (R1 GTGTCTTGCCCCTCTCCTCCCCAAGCC 3') AND 9410-9386 (R3, 5' GAGAGACTACTCAGGGAGAGCCTCC 3').

Reaction mixtures had a total volume of 100 μl and contained 400 Ng of genomic DNA, 0.3 μM of each synthetic oligonucleotide primer (see previous paragraph), 200 μM each of dATP, dGTP, dCTP and TTP, 50 mM KCl, 10 mM Tris (pH 8.0), 1.5mM $MgCl_2$, 0.001% gelatin and 2.5 units of Taq polymerase. The samples were overlayered with mineral oil and placed in a Perkin Elmer Cetus thermocycler. Template DNA was denatured at 94°C, 30 second DNA annealing period at 62°C and a 2 minute DNA extension period at 72°C was performed and 50 μl of the PCR products were electrophoresed in a polyacrylamide gel and stained with ethidium bromide (see Figure 4).

Results:

Using the 1337-1363, 9410-9386 primers PCR products of ~ 1350 bp are obtained from chromosomes bearing GH1 gene deletions but no are formed from normal chromosomes (see Figure 4). No product results from non-deleted chromosomes because the R1 primer anneals only to R1 and the R3 primer only t6o R 3 the normal distance between these (~9.4 kb) on non-deleted chromosomes prevents the formation of PCR products. Since the 1350 bp fragments are only synthesized in the case of a GH1 gene deletion only gel electrophoresis is needed after PCR amplification and restriction enzyme digestion is no longer required.

Experiment 4

Materials and Methods

The human prion gene has been mapped to 20p12-pter and encodes a protein, whose function has not yet been defined. Th structure of the gene is unusual in that it has a single open reading frame approximately 808 base pairs (bp) in length. Interestingly, a 24 base pair sequence is tandemly repeated 5 times within this region (Kretzschmar et al (1986) DNA 5:315-324).

By virtue of their repetitive nature, these sequences may participate in unequal homologous recombination that results in the loss of gain of a repetitive unit or part thereof. Using the polymerase chain reaction (PCR) and oligonucleotide primers and amplification conditions originally described by Hsiao, et al. (1989) Nature 338:342-345, the human prion gene may successfully be amplified. Following polyacrylamide gel electrophoresis the products may be analyzed for a deletion (see Figure 6). The 860 bp fragments represent the expected gene product using the primers and conditions originally described by Hsiao et al. (1989) Nature 338:342-345. The 835 bp fragments represent products from an altered allel containing a 24 bp deletion (see Figure B- Kretzschmar et al. (1986) DNA 5:315-324). The heteroduplex fragments represent the annealing of PCR amplified fragments derived from the normal and altered allel (Bosque, P.J., Vnencak-Jones, C.L., Johnson, M.D. and McLean, J.J. (1991) in preparation for submission to Neurology). The ligonucleotide primers required for this amplification are: 5' AAGGATCCCTCAAGCTGGAAAAAGA 3' AND 5' AAGAA TTCTCTGACATTCTCCTCTTCA 3'. The

amplification conditions included an initial denaturation at 94°C for six minutes, followed by 35 cycles of 94°C for ninety seconds, 50°C for ninety seconds and 72°C for three minutes. Extension of the amplified segments were completed at 72°C for ten minutes.

Results:

Familial Creutzfeld-Jakob disease (FCJD) is a rare ($<10^{-6}$) autosomal dominant disorder. The delayed onset of ataxia and/or dementia is followed by death often within two years. FCJD is confirmed by pathologic brain findings. Alterations in the prion gene (20p 12→pter) have been demonstrated in some cases of FCJD.

To determine the status of prion genes in a three generation kindred with five affecteds, the open reading frame of the prion gene was PCR amplified (see Figure 6). Acrylamide gels revealed the ~860 bp fragments seen in controls as well as an ~835 bp homoduplex and 2 heterduplex bands. Sequence analysis showed nucleotides 295-319 (codons 83-90) containing the final of five octapeptide tandem repeats within the expressed protein were deleted. This repetative modify may predispose

to unequal homologous recombination analogous to that producing GH gene deletions. This repetative prion open reading frame may predispose to unequal homologous recombination analogous to that producing GH gene deletions.While DNA was unavailable from the other affected individuals (deceased), all had either an affected offspring or asymptomatic, heterozygous offspring (<40 years). Interestingly, when the PCR products of 166 control chromosomes were examined, 5/238 (2.1%) contained a similar size prion gene deletion. This finding could result from recurring unequal recombination events ofaffects the prion gene. Heteroduplexes formed after mixing PCR products of each of these with those of the proband suggested that 3/5 deletions may be identical to that of the proband. However, the proband also may be identical to that of the proband. However, the proband also had a G to A substitution at nucleotide 581 corresponding to a CpG dinucleotide that results in an Asp to Asn substitution at codon 178.

The data indicate that a prion allele with a partial deletion occurs in a proband and segregates with FCJD in his kindred but may also

occur in controls. The role, of this additional nucleotide substitution in the prion allele in causing FCJD is under ongoing investigation.

Experiment 5

Materials and Methods

Genomic DNA was isolated, as previously described from individuals with familial orthostatic intolerance and controls.

DNAs were PCR amplified in 100 μl reactions containing mixtures of 200 μg of genomic DNA, 0.5 μM of synthetic oligonucleotide primers 167-191 5' CACGGCGGTGAGATAACCAAGGAC 3' and 2552-2526 5'CCAACGCAGGCATTTGTCTTCTGTCC 3' (see prion gene sequence), 200 μM of each dNTP, 50 mM KCl, 10 mM (Tris pH8.0), 1.5 mM $MgCl_2$, 0.001% gelatin and 2.5 units of TaqI polymerase. The reactions were placed in a thermal cycler, denatured at 94°C for six minutes, then subjected to 30 amplification cycles. Each cycle consisted of a sixty second DNA denaturation period at 94°C, as a 120 second annealing period at 56°C and a 120 second DNA extension period at 72°C. After the 30 cycles the samples were allowed to anneal for ten minutes at 72°C, then cooled to 0°C.

Following PCR amplification, 25 μl of each reaction mixture were digested with the restriction endonucleases MspI, DdeI, HaeIII or RsaI using conditions specified by the commercial suppliers. The resulting DNA fragments were then subjected to electrophoresis in 5% polyacrylamide gels and visualized by ethidium bromide staining (see Figure 5).

Results:

Atrial natriuretic factor (ANF) is a small peptide hormone synthesized by atrial cardiomyocytes. Infusion of ANF in man causes decreased arterial pressure due to decreased cardiac output and vascular resistance and decreased intravascular volume. Individuals with familial orthostatic intolerance (FOI) development hypotension and dizziness on standing and may have mitral valve proplapse. Interestingly, individuals in certain FOI kindreds have paradoxical increases in ANF levels on standing suggesting possible abnormalities in ANF or in its clearance receptor. An intragenic ANF PCRFLP was identified to map ANF and study its linkage relationship to FOI. HhaI digestion of a 2.552 kb PCR fragment containing the entire

ANF gene detected a RFLP whose major allel was 0.81. Using the CEPH panel of DNAs, ANF was found to be linked to CFI-C52 (θ =.18, LOD = 3.77), CRI-L336 (θ =.20, LOD = 3.4) and tissue fucosidase α-L-1 (θ = .13, LOD - 4.2). In a multiplex FOI kindred with apparent autosomal recessive inheritance the 2 affected sibs and 1 non-affected sib were condordant and discordant, respectively, for maternal ANF alleles. The data 1) confirm the assignment of the ANF locus to 1p36 and identify multiple linked markers, and 2) raise the possibility that derangements of ANF may contribute to FOI.

Applicant finding additional, smaller fragments following digestion of the same PCR products with DdeI, HaeIII or RsaI (see Figure 5) is best explained by the presence of a small deletion in one ANF allel. Such a deletion could only occur by unequal recombination between Alu related sequences that occur in 3' end of the ANF gene. Such inter Alu recombinations could truncate the size of one Alu repeat thereby yielding slightly smaller sizes when PCR products contianing ANF are digested with multiple restriction endonucleases (see Figure 5).

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

What is claimed is:

1. A method of detecting gene deletions, said method including the steps of: (1) obtaining a genomic DNA sample, the sample normally including gene having two ends normally flanked by two homologous regions having substantially identical portions the gene deletion being in the form of a fusion fragment of the two homologous regions; (2) amplifying the genomic DNA using a set of primers capable of amplifying both the fusion fragment and the two homologous regions having the gene in-between; and (3) characterizing the amplified fusion fragments alone indicating a homozygous deletion, a combination of fusion fragments and the two homologous regions and the gene in-between indicating a heterozygote deletion, or the two homologous regions and the gene alone indicating a homozygous nondeletion, at least one of the homologous regions contains a site susceptible to cleavage by a particular enzyme, said characterizing step being further defined as enzymatically digesting the amplified genomic DNA with the particular enzyme, producing digestion fragments from the fusion fragments having

lengths different from digestion fragments of the two homologous regions having the gene in-between and identifying the samples including deletions based on observing the different size digestion fragments.

2. A method as set forth in claim 1 wherein said amplifying step is further defined as polymerase chain reaction amplifying the genomic DNA sample.

3. A method as set forth in claim 2 wherein said amplifying step is further defined as using a set of primers complementary to the substantially identical portion of the two homologous regions enabling the primers to amplify either the fusion fragment or the two homologous regions having the gene region in-between.

4. A method as set forth in claim 1 wherein said amplifying step is further defined as using a primer set having the sequences (a) 5'TCCAGCCTCAAAGAGCTTAC3' and (b) 3'AGGTAACGAGTTCCGAGACCCTT5'.

5. A method as set forth in claim 4 wherein each primer sequence of the primer set includes a nucleotide triplet at each 5' end to facilitate cloning of the polymerase chain reaction amplified products.

6. A method as set forth in claim 5 wherein primer sequence (a) includes the triplet 5'GGA3' flanking the 5' end thereof and primer sequence (b) includes the triplet 3'AAG5' flanking the 3' end thereof.

7. A method as set forth in claim 1 wherein said identifying step is further defined as electrophoretically separating the produced fragments and visually observing the separated fragments.

8. A method as set forth in claim 1 wherein the deletion is of the GH1 gene from the R1 region of the DNA fragment EcoRI, EcoRI containing two homologous flanking regions R1 and R3, the R3 region including two SmaI sites, said amplifying step being further defined as amplifying 1918 bp fusion fragments from a homozygote for the GH1 deletion, 1900 and 1921 bp

fragments from a nondeletion control, and a combination of 1918 bp and 1900 bp and 1921 bp fragments from a heterozygote, said producing step being further defined as producing 1900, 761, 712 and 448 bp digestion fragments from the control, 1470 and 448 bp digestion fragments from the homozygote, and the combination of 1900, 761, 712 and 448 bp with 1470 and 448 bp digestion fragments from the heterozygote.

9. A primer set for detecting gene deletions in a genomic DNA sample possibly including a gene deletion, the gene having two ends normally flanked by two homologous regions having substantially identical portions, the gene deletion being in the form of a fusion fragment of the two homologous regions, said primer set including nucleotides complementary to the substantially identical portions of the two homologous regions for enabling said primers to amplify the fusion fragment or the two homologous regions having the gene region in-between.

10. A primer set as set forth in claim 9 wherein said set of primers have the sequence

(a) 5'TCCAGCCTCAAAGAGCTTAC3' and (b) 3'AGGTAACGAGTTCCGAGACCCTT5'.

11. A primer set as set forth in claim 10 wherein said primer sequences each include a nucleotide triplet at the 5' end thereof to facilitate cloning of polymerase chain reaction amplified products.

12. A primer set as set forth in claim 11 wherein said primer sequence (a) includes a triplet 5'GGA3' flanking said 5' end thereof and said primer sequence (b) includes a triplet 3'AAG5' flanking said 3' end thereof.

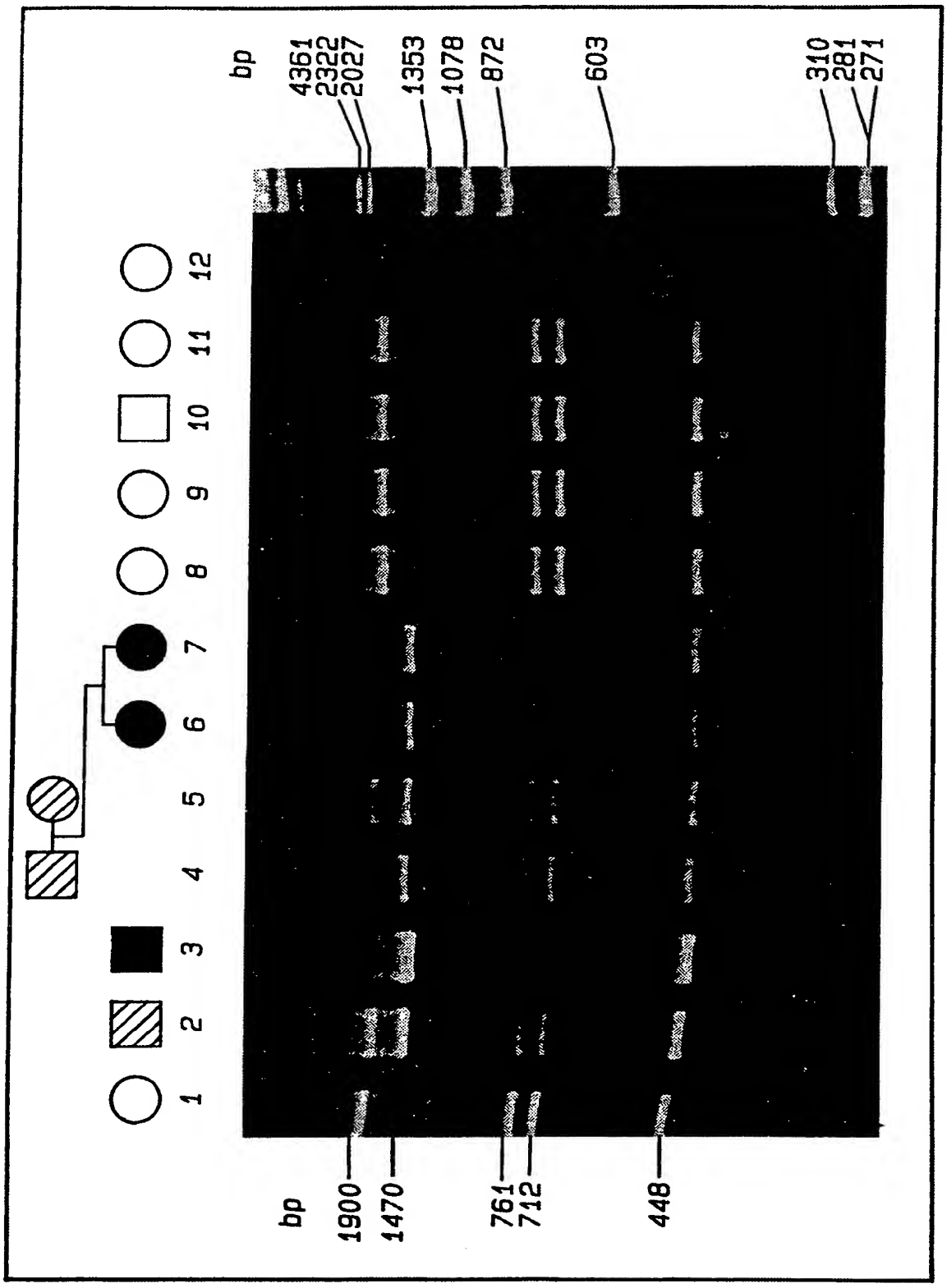

Fig-1

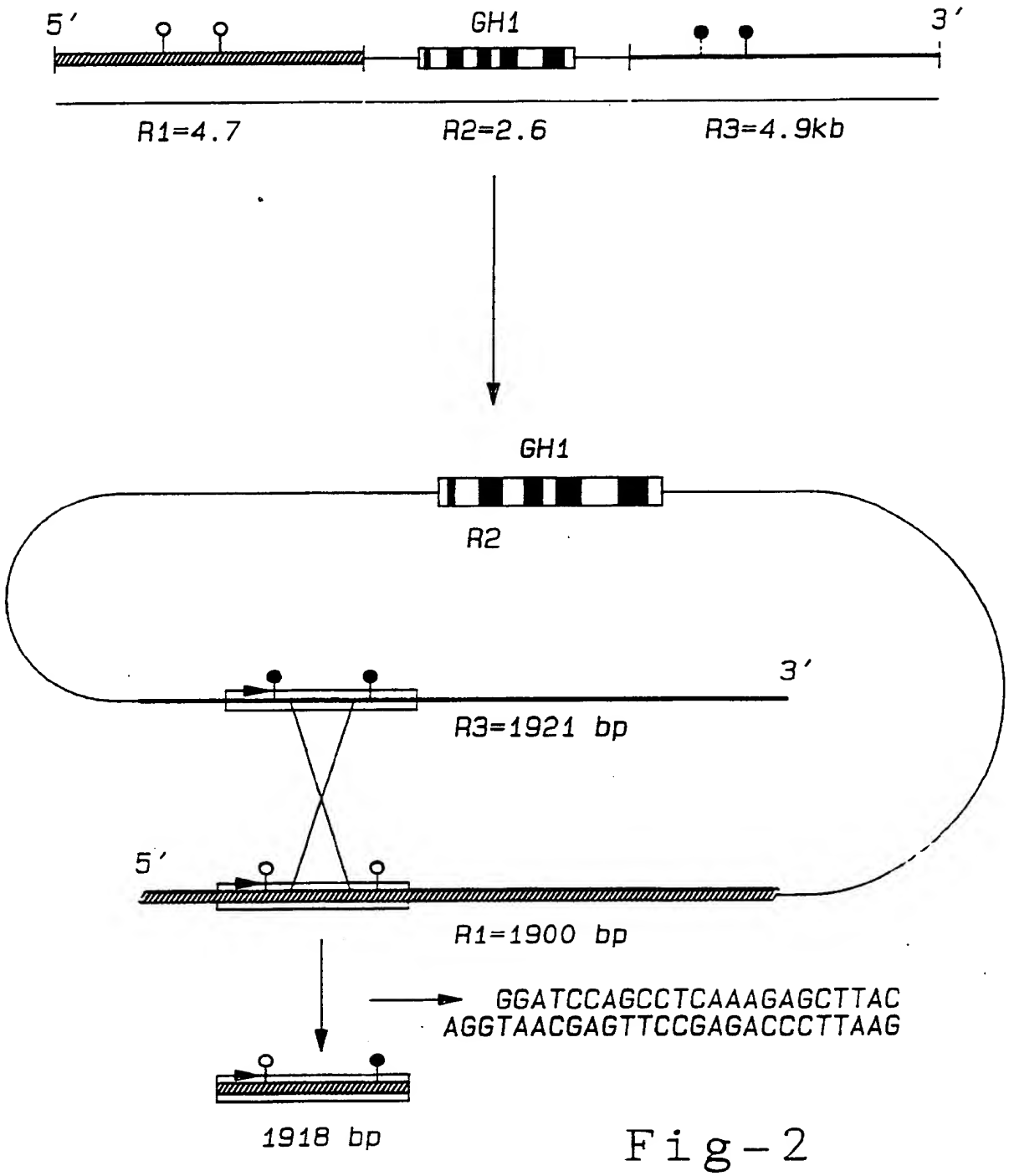
5′
GH1
3′
R1=4.7
R2=2.6
R3=4.9kb
GH1
R2
3′
R3=1921 bp
5′
R1=1900 bp
GGATCCAGCCTCAAAGAGCTTAC
AGGTAACGAGTTCCGAGACCCTTAAG
1918 bp

Fig-2

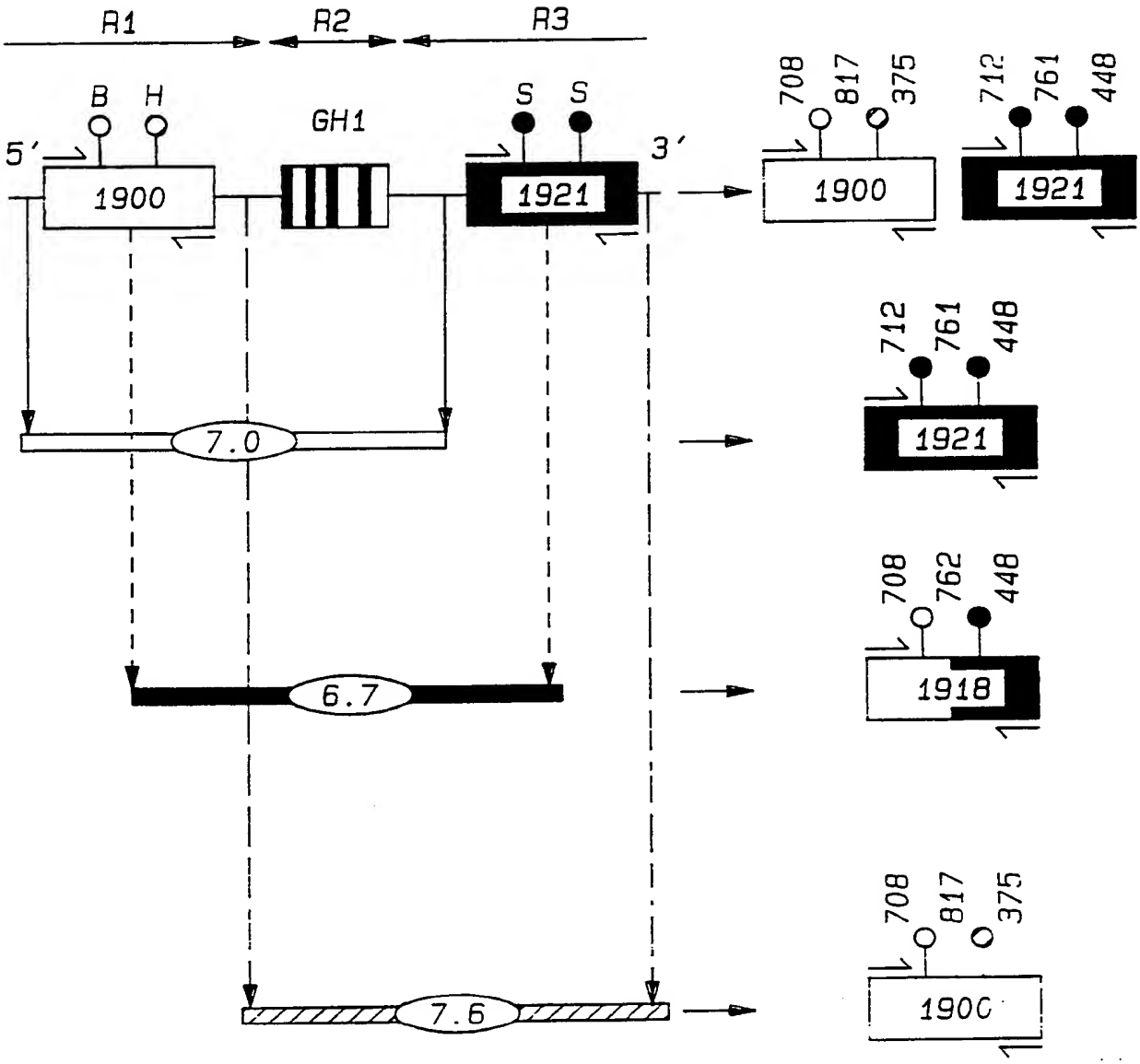
R1
R2
R3
B
H
GH1
S
S
5′
3′
1900
1921
708
817
375
712
761
448
7.0
6.7
7.6
762
1918
1900

Fig-3

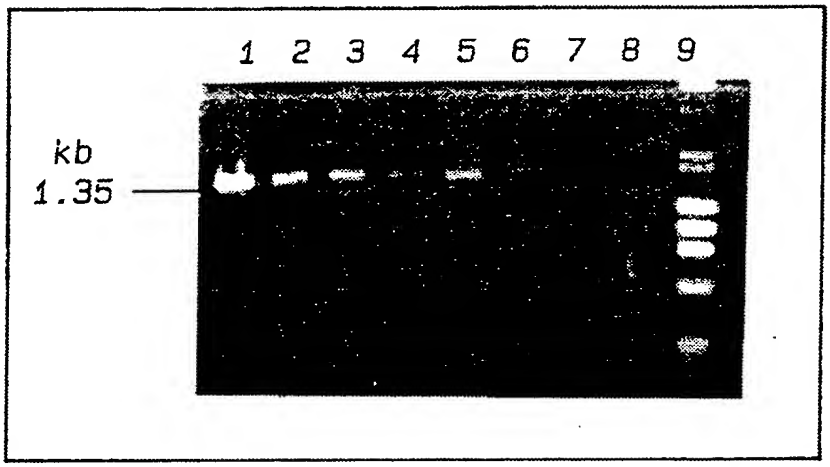

Fig-4

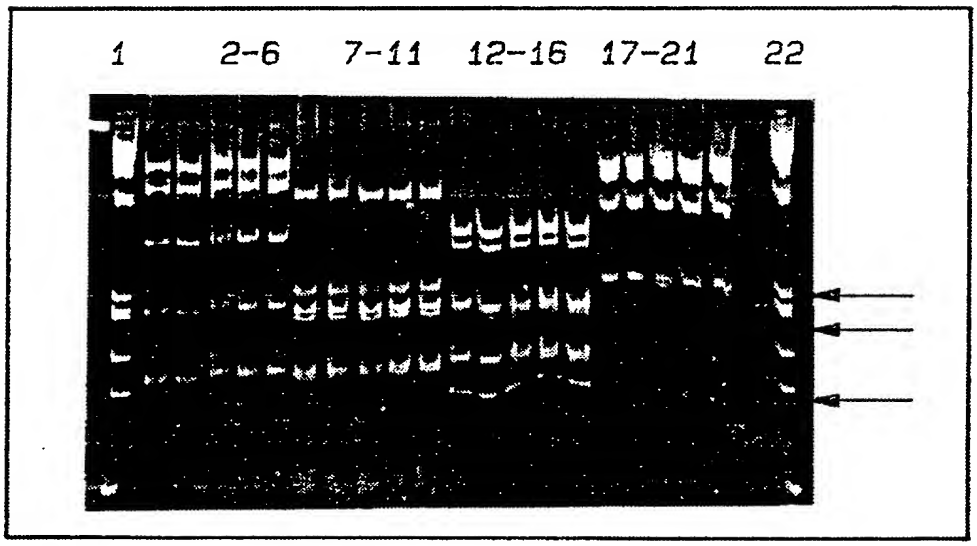

Fig-5

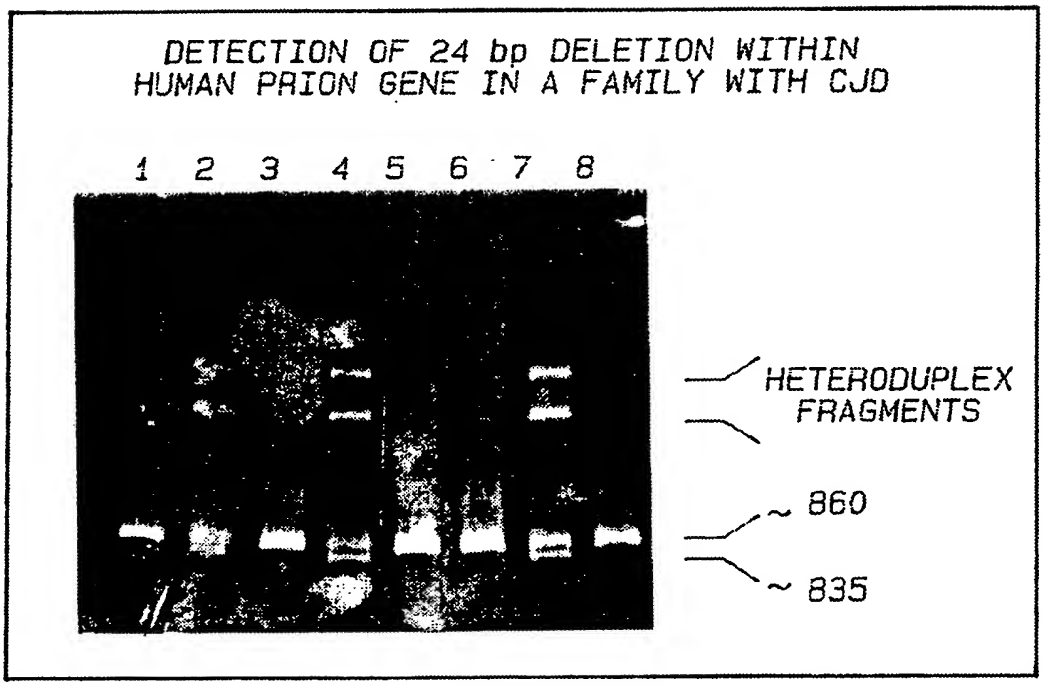

Fig-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04764

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34; G01N 33/566, 33/48; C07H 15/12
US CL :435/6, 91; 436/501,94; 536/26, 27, 28, 29; 935/77, 78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 436/501,94; 536/26, 27, 28, 29; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG:Biosis, Biotech. Abstr., WPI, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, USA, Vol. 85, Issued August 1988, Vnencak-Jones et al., "Molecular Basis of Human Growth Hormone Gene Deletions", pages 5615-5619, see entire document	1-12
Y	The Journal of Biological Chemistry, Vol. 266, No. 13, Issued 05 May 1991, S-H. Yoon et al., "Molecular Defect of Truncated Beta-Spectrin Associated with Hereditary Elliptocytosis", pages 8490-8494, see last eleven lines of abstract	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 SEPTEMBER 1992	17 SEP 1992
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